DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts

Karouzakis, E ; Rengel, Y ; Jüngel, A ; Kolling, C ; Gay, R E ; Michel, B A ; Tak, P P ; Gay, S ; Neidhart, M ; Ospelt, C

Abstract: In the search for specific genes regulated by DNA methylation in rheumatoid arthritis (RA), we investigated the expression of CXCL12 in synovial fibroblasts (SFs) and the methylation status of its promoter and determined its contribution to the expression of matrix metalloproteinases (MMPs). DNA was isolated from SFs and methylation was analyzed by bisulfite sequencing and McrBC assay. CXCL12 protein was quantified by enzyme-linked immunosorbent assay before and after treatment with 5-azacytidine. RASFs were transfected with CXCR7-siRNA and stimulated with CXCL12. Expression of MMPs was analyzed by real-time PCR. Basal expression of CXCL12 was higher in RASFs than osteoarthritis (OA) SFs. 5-azacytidine demethylation increased the expression of CXCL12 and reduced the methylation of CpG nucleotides. A lower percentage of CpG methylation was found in the CXCL12 promoter of RASFs compared with OASFs. Overall, we observed a significant correlation in the mRNA expression and the CXCL12 promoter DNA methylation. Stimulation of RASFs with CXCL12 increased the expression of MMPs. CXCR7 but not CXCR4 was expressed and functional in SFs. We show here that RASFs produce more CXCL12 than OASFs due to promoter methylation changes and that stimulation with CXCL12 activates MMPs via CXCR7 in SFs. Thereby we describe an endogenously activated pathway in RASFs, which promotes joint destruction.

DOI: https://doi.org/10.1038/gene.2011.45

Originally published at:
Karouzakis, E; Rengel, Y; Jüngel, A; Kolling, C; Gay, R E; Michel, B A; Tak, P P; Gay, S; Neidhart, M; Ospelt, C (2011). DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts. Genes and Immunity, 12(8):643-652.
DOI: https://doi.org/10.1038/gene.2011.45

DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts

Emmanuel Karouzakis[1], Yvonne Rengel[1], Astrid Jüngel[1], Christoph Kolling[2], Renate E. Gay[1], Beat A. Michel[1], Paul P. Tak[3] Steffen Gay[1], Michel Neidhart[1], Caroline Ospelt[1,3]

[1]Center of Experimental Rheumatology and Zurich Center of Integrative Human Physiology (ZIHP), University Hospital Zurich, Switzerland

[2]Schulthess Clinic, Zurich, Switzerland

[3]Department of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Corresponding author: Emmanuel Karouzakis

Gloriastrasse 23

CH-8091 Zurich

Switzerland

Tel.: +41 44 255 5866

Fax: +41 44 255 4170

emmanuel.karouzakis@usz.ch

Keywords: CXCL12, rheumatoid arthritis synovial fibroblasts, epigenetics, DNA methylation

ABSTRACT

In the search for specific genes regulated by DNA methylation in RA, we investigated the expression of CXCL12 in SF and the methylation status of its promoter and determined its contribution to the expression of matrix metalloproteinases (MMPs).

DNA was isolated from SF and methylation was analysed by bisulphite sequencing and McrBC assay. CXCL12 protein was quantified by ELISA before and after treatment with 5-azacytidine. RASF were transfected with CXCR7-siRNA and stimulated with CXCL12. Expression of MMPs was analysed by Real-Time PCR.

Basal expression of CXCL12 was higher in RASF than osteoarthritis (OA) SF. 5-azacytidine demethylation increased the expression of CXCL12 and reduced the methylation of CpG nucleotides. A lower percentage of CpG methylation was found in the CXCL12 promoter of RASF compared to OASF. Overall, we observed a significant correlation in the mRNA expression and the CXCL12 promoter DNA methylation. Stimulation of RASF with CXCL12 increased the expression of MMPs. CXCR7 but not CXCR4 was expressed and functional in SF.

We show here that RASF produce more CXCL12 than OASF due to promoter methylation changes and that stimulation with CXCL12 activates MMPs via CXCR7 in SF. Thereby we describe an endogenously activated pathway in RASF which promotes joint destruction.

1. INTRODUCTION

A variety of studies describe an activated phenotype of synovial fibroblasts (SF) in rheumatoid arthritis (RA) which is characterized by a changed morphology, deranged apoptotic behavior, and increased invasive properties [1]. Recently, we found that epigenetic changes might contribute to these phenotypic changes in RASF. We could show that DNA of RASF is globally hypomethylated when compared to osteoarthritis (OA) SF or normal SF and that less DNA methyltransferase DNMT1, the enzyme responsible for DNA de novo methylation is present in RASF [2]. DNA methylation is well known to regulate gene expression. Transcriptional regulation by DNA methylation occurs in CpG rich regions of gene promoters, so called CpG islands. Methyl CpG binding proteins bind to methylated CpG islands and together with chromatin remodeling enzymes cause gene silencing [3]. In the normal genome, DNA methylation regulates gene expression to form tissue specific expression patterns [4]. Aberrant DNA methylation was found in various pathologies including cancer and autoimmune diseases [5, 6].

CXCL12, also known as stromal-derived factor-1 (SDF-1) is a key player in the trafficking of lympho- and hematopoietic progenitor cells and in the early development and regeneration of tissues [7]. Moreover, the secretion of CXCL12 is increased after tissue damage by hypoxia, toxins or irradiation, leading to the immigration of progenitor cells expressing the CXCL12 receptor CXCR4 [8]. A second receptor for CXCL12, CXCR7 has only most recently been identified and has been implicated in tumor growth and metastasis [9-11]. Elevated levels of CXCL12 were found in patients with multiple sclerosis, inflammatory myopathies, spondyloarthropathies and RA [12-17]. Levels of CXCL12 in the synovial fluid of RA patients are around 10 times higher than in healthy joints and reach a mean of 750 ng/ml [16]. It is understood that CXCL12 drives chronic inflammation by attraction of monocytes and lymphocytes into the joint and by stimulation of synovial fibroblasts to produce pro-

inflammatory cytokines [17-20]. Previous studies also showed that cultured RASF produce more CXCL12 than normal or OA synovial fibroblasts [16, 21].

Based on these results, we analyzed in the current work whether expression of CXCL12 in RASF is modified by changes in DNA methylation. Furthermore, to elucidate CXCL12 signaling pathways we looked at the expression of CXCR7 in synovial fibroblasts and tested whether CXCR7 mediates the production of matrix-metalloproteinases (MMPs) after CXCL12 stimulation in RASF.

2. MATERIAL AND METHODS

2.1 Patients and tissue preparation: Synovial tissues were obtained from trauma patients (normal synovial fibroblasts, NSF, n=2), RA n=14 and OA n=11 patients undergoing joint replacement surgery at the Schulthess Clinic Zurich after written consent (Table 1). All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [22]. Normal lung fibroblasts were isolated from open lung biopsies of patients suspected with cancer but with a negative diagnosis. Synovial fibroblasts and normal lung fibroblasts were isolated and cultured as described previously [23].

2.2 Bisulphite sequencing: Genomic DNA was prepared from synovial fibroblasts using the QiAmp DNA blood Mini kit (Qiagen, Hombrechtikon, Switzerland). The DNA (1µg) was bisulphite modified using the EpiTect bisulphite kit (Qiagen). Two rounds hemi-nested PCR amplification of bisulphite modified DNA (2 µl) was performed using the AmpliTaq Gold polymerase (Applied Biosystems, Rotkreuz, Switzerland). The PCR cell cycle program was 95°C 4 min ; 95°C 30s, 52°C 90 sec, 72°C 2 min x 5 ; 95°C 30sec, 52°C 90sec, 72°C 90 sec x 25 ; 72°C 4 min. Primers were designed for two regions of the CXCL12 CpG island promoter. The forward 5-GTT TGT GAT TAG TTT ATT TTA TTA-3, reverse 5-CTA AAT AAA AAC CAA TAA AAA AC-3 and hemi-reverse 5-AAA AAA TCC TAC TTT CTA TAC-3 bisulphite sequencing primers amplified the region -741bp to -477bp and the forward 5-GTT

TTT TAT TGG TTT TTA TTT AGT TTT-3, reverse 5- TAC CTC CAC CCC CAC TAT AT-3 and hemi reverse 5- GAG TTT GAG AAG GTT AAA GGT-3 bisulphite sequencing primers amplified the region -244bp to +272bp. The computer software MethPrimer predicted CpG islands with the following criteria: island size >100, GC content > 50 % and Obs/Exp >0.6 and designed bisulphite primers for DNA methylation analysis [24]. The PCR purified fragments were cloned using the Qiagen PCR cloning handbook according to manufacturer instructions (Qiagen). Positive clones were sequenced (Microsynth, Balgach, Switzerland). The data was analysed using the BiQ analyzer software [25].

2.3 McrBC digestion and quantitative Real-time PCR: The CXCL12 promoter DNA methylation was determined by methylation-sensitive McrBC-PCR assay. Genomic DNA (1µg) was sonicated using the Diagenode bioruptor (10 min, 15 sec on and 15sec off). The sonicated DNA was digested with 10 units of McrBC or mock undigested in a 50µl reaction mixture containing 1x NEB2 buffer, 10mM GTP and 0.1mg/ml BSA. The restriction digestion reaction was incubated at 37 ^{o}C overnight and then the reaction was deactivated with heating at 65 ^{o}C for 20 min. The McrBC treated DNA and mock samples were purified using Qiagen PCR purification protocol kit. Quantitative SYBR green PCR using the CXCL12 promoter (-741 to -477) primers: Forward CAC CAT TGA GAG GTC GGA AG, Reverse AAT GAG ACC CGT CTT TGC AG, was carried out with the McrBC digested and mock samples. McrBC cleaves methylated DNA strands and inhibits PCR amplification. In contrast, unmethylated DNA prevents McrBC cleavage and can be measured by quantitative PCR. Mock undigested DNA is considered as total amount of DNA used in the reaction. Methylated DNA will have decreased Ct values after McrBC digestion. The Real-time PCR results were normalized with the mock treated sample DNA (deltaCt) and presented as fold induction of PCR recovery after digestion with McrBC.

2.4 Stimulations: RASF and OASF were seeded at low density 24h before treatment. RASF were stimulated with 100 ng/ml recombinant CXCL12 (Peprotech, London, UK). OASF were

treated with 0.5 μM or 1 μM 5-azacytidine (Sigma) for 6 days. The cell culture medium was changed every 24h and replaced with new 5-azaC.

2.5 Transfection of CXCR7 small interfering RNA (siRNA): siRNA for CXCR7 and double stranded siRNA without homology to mammalian genes (negative control) (both from Qiagen) were used for silencing experiments. Also, transfection without siRNA (mock) was performed to determine whether the experimental set-up causes unspecific effects. The transfections were done by electroporation (Nucleofector, Lonza, Cologne, Germany) using transfection reagents for primary mammalian fibroblasts (Basic Nucleofector® Kit; Lonza) at a concentration of 0.9 μg siRNA/5x10^5 cells. Transfected RASF were cultured for 48h before efficiency of knock-down was measured by Real-time PCR, FACS and Western blot or before stimulations were begun.

2.6 RNA isolation and quantitative Real-time PCR: Total RNA was isolated using the RNeasy Miniprep kit (Qiagen) including DNase treatment. RNA was reverse transcribed using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). Samples without addition of reverse transcriptase served as negative control (non RT). Relative quantification of mRNA levels by TaqMan®/SYBRGreen® Real-time PCR was done using eukaryotic 18S ribosomal RNA as endogenous control (Applied Biosystems). The differences of the comparative threshold cycles (Ct) of sample and 18S cDNA were calculated (dCt). Relative expression levels were calculated following the formula ddCt = dCt (sample stimulated) - dCt (sample unstimulated). Relative expression was calculated using the expression 2^{-ddCt}. The sequence of the primers used for measuring MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, TIMP-1, TIMP-2, TIMP-3 were previously described [26].

2.7 PCR: mRNA was reverse transcribed using oligo dT and Moloney Murine Leukemia Virus reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). PCR was performed on a C1000™ Thermal Cycler (Bio-Rad laboratories, Hercules, CA) with the

following primer pairs and protocols designed to detect the 4 different CXCR4 mRNAs as published on the National Center for Biotechnology Information AceView database. CXCR4 transcript 1: forward primer GGAAAAGATGGGGAGGAGAG, reverse primer CACTTCCAATTCAGCAAGCA; CXCR4 transcript 2: forward primer CAGCAGGTAGCAAAGTGACG, reverse primer GTAGATGGTGGGCAGGAAGA; CXCR4 transcript 3: forward primer AAGGGTCACCGAAAGGAGTT, reverse primer GAAGAGACCGGTGGTCTGAG; CXCR4 transcript 4: forward primer GTTAAGCGCCTGGTGACTGT, reverse primer GGTAACCCATGACCAGGATG. 94°C 5min, 40 cycles with 94°C 30s, 58°C 30s, 72°C 30s and a final elongation of 5min with 72°C. β-microglobulin: forward primer AAGATTCAGGTTTACTCACGTC, reverse primer TGATGCTGCTTACATGTCTCG. 94°C 5min, 35 cycles with 94°C 30s, 55°C 30s, 72°C 30s, final elongation 72°C 5min. Reaction products were separated on a 1.5% agarose gel and signals were visualized using ethidium bromide.

2.8 Western blot analysis: Whole cell lysates were prepared by lysing cells in 2x Laemmli buffer. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). Membranes were blocked for 1h at room temperature in 5% nonfat dry milk with 0.05% Tween 20 in TBS and were incubated overnight with polyclonal rabbit anti-human CXCR7 (2.8 µg/ml; Abcam, Cambridge, UK). To ensure specificity of the CXCR7 antibodies, blots were incubated with CXCR7 antibodies which were pre-incubated for 1h at 37°C with or without 10 µg/ml of a synthetic CXCR7 peptide (10 µg/ml; Abcam). Afterwards, membranes were incubated with HRP-conjugated secondary antibodies (Jackson Immunoresearch, Suffolk, UK). Bound antibodies were visualized using enhanced chemiluminescence (Amersham Bioscience, Otelfingen, Switzerland). Equal protein loading was confirmed using mouse anti-human α-tubulin antibodies or Ponceau solution (Sigma-Aldrich, Basel, Switzerland).

2.9 Flow Cytometry: RASF were detached from the culture flask with accutase (PAA Laboratories, Linz, Austria), washed with PBS/1%FCS and incubated with 20 µg/ml of rabbit anti-human CXCR7 (Abcam, Cambrige, UK) or of rabbit IgG for 45 minutes at 4°C. Cells were washed with PBS/1%FCS and subsequently incubated with FITC-labeled goat anti-rabbit IgG for 45 minutes at 4°C. Washing steps were repeated twice before analysis of the cells in the flow cytometer (FACSCalibur). Data were processed using CellQuest software (BD Biosciences, San Diego, CA).

2.10 ELISA: Synovial fibroblasts were seeded at 1 x 10^5/ml DMEM and CXCL12 was measured after 24h (Figure 1B) or at 2 x 10^5/ml DMEM and CXCL12 was measured after 24h and 96h (Figure 5C) in the supernatants with the DuoSet® ELISA development system (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

2.11 CXCL12 blockade: RASFs (2 x 10^5) were incubated with 1 µg/ml neutralizing anti-human CXCL12 antibodies (R&D Systems) or IgG control. Fresh antibodies or control was added after 48h.Cells were lysed after 24h or 96h.

2.12 Statistical analysis: All data are expressed as mean ±SEM. Statistical analysis was performed using GraphPad Prism software, version 4.03 (GraphPad System, San Diego, CA). For analysis between different groups, the Mann-Whitney U, for paired analysis Wilcoxon signed rank test was used. *P* values less than 0.05 were considered significant.

3. RESULTS

3.1 RASF upregulate CXCL12 mRNA and protein expression.

To analyse the basal expression of CXCL12 expression in RASF and in OASF, we measured CXCL12 mRNA and protein levels in cell culture supernatants after 24h. RASF express significantly more CXCL12 mRNA than OASF and NSF (RASF 6.9±0.3 dCt, n=11; OASF 8.3±0.5 dCt, n=8; NSF 7.5 dCt, n=1; $p<0.028$) (Fig 1A). Furthermore, we observed that the mRNA expression of CXCL12 was positively correlated with the C-reactive protein, a marker

of inflammation marker in these patients (r =-0.64, p<0.01). RASF produced with 85±12 pg/ml also significantly more CXCL12 protein than OASF with 52 ±10 pg/ml (Fig.1B).

3.2 DNA methylation regulates the promoter of CXCL12

To investigate whether changes in DNA methylation might influence expression of CXCL12 in RASF, we first tested whether the CXCL12 promoter can be regulated by DNA methylation. Computational analysis predicted that there are CpG islands upstream of the transcription initiation site of the CXCL12 promoter (Fig. 2A). We further analysed the region that is 100 bp upstream of exon 1 (-741bp to -477bp) and the region that spans exon 1 (-244bp to +272bp) by bisulphite sequencing. Our findings showed that NSF have a high percentage of methylated CpG nucleotides in the promoter upstream region from -741bp to -477bp, while in the region -244 to +272, that spans the transcriptional initiation site and exon 1, all the CpG nucleotides were unmethylated (Fig. 2B). To see whether demethylation of the heavily methylated CpG rich region would influence CXCL12 expression, we treated OASF with different doses of the demethylating drug 5-azaC and found dose dependent upregulation of CXCL12 protein secretion (Fig. 2C). Furthermore, bisulphite sequencing analysis showed demethylation of the CXCL12 promoter (-741bp to -477bp) with 1 μM 5-azaC (Fig. 2D). In addition, the mRNA expression of CXCL12 correlated with the methylation levels of the CXCL12 promoter (r =0.60, p<0.04) as show my McrBC digestion assay (Fig. 2E).Taken together, the results of figure 2 indicate that expression of CXCL12 is regulated by methylation of its promoter, and accordingly can be increased by demethylation.

3.3 Demethylation of CXCL12 promoter upregulates CXCL12 mRNA expression.

To investigate whether the methylation status of the CXCL12 promoter is altered in fibroblasts of RA patients, we compared the percentage of CpG methylation between RASF NSF, OASF and normal lung fibroblasts (Fig. 3A, B). First, a group of 4 pooled RASF

patients and a group of 3 pooled OASF patients were compared. The analyzed promoter region of CXCL12 had significantly less CpGs methylated in the RASF group than in the OASF group (RASF 21% ± 7.2 versus OASF 42% ±7.8). We then analysed whether the results from the pooled groups would be reflected in individual patient's analysis. CXCL12 promoter had similar changes as seen in the pooled samples (RASF 1-2 27% ± 4.8 versus OASF 1-2 46% ± 5.6). The CXCL12 promoter in NSF cell cultures was strongly methylated (NSF1 54% and NSF2 60%). As expected, the CXCL12 promoter in lung fibroblasts was similarly strongly methylated as in NSF or OASF. The above results suggest that normally the expression of CXCL12 is repressed by methylation in fibroblasts, but that an epigenetic defect in synovial fibroblasts of RA patients causes an intrinsically increased expression of CXCL12.

3.4 Overexpression of CXCL12 induces functional changes in RASF

Stimulation of synovial fibroblasts, chondrocytes and osteoclasts with CXCL12 in vitro was previously found to induce the expression of matrix degrading enzymes [16, 27, 28]. Since DNA methylation induces CXCL12 overexpression, we measured mRNA expression of the collagenases MMP-1 and MMP-13, the gelatinases MMP-2 and MMP-9, stromelysin (MMP-3), and the matrix-bound MMP-14 after stimulation with CXCL12 for 24h in RASF. In addition, we looked at the mRNA expression of the tissue inhibitors of matrix metalloproteinases (TIMPs) -1, -2, and -3. . CXCL12 levels in joints of RA patients have been measured to be on average 375 and 750 ng/ml respectively[16, 29]. We stimulated RASFs in vitro with 100 ng/ml. Stimulation with CXCL12 selectively and significantly increased the expression of MMP-1 by 6 ±3 fold, of MMP-3 by 2 ±0.2 fold and of MMP-13 by 4 ±1 fold (Fig.4A). In contrast, CXCL12 neither influenced the expression of MMP-2, MMP-9, or MMP-14, nor did it change the expression levels of TIMPs.

To see whether endogenous production of CXCL12 by RASFs in culture would be enough to stimulate the expression of MMPs, we increased the concentration of CXCL12 in the cell culture supernatants by increasing the ratio between seeded cells and medium and by prolonging incubation times and added an CXCL12 neutralizing antibody or IgG control (Fig. 4B). Expression of MMP-1 was decreased 26% after 24h, and 42% after 96h incubation by CXCL12 blockade compared to IgG control.

We then addressed the question whether CXCL12 signals via CXCR4 or the newly found CXCL12 receptor CXCR7 in synovial fibroblasts. Transcription of the CXCR4 gene produces 4 different mRNAs which putatively encode 4 different isoforms of the protein. We analyzed the expression of these 4 transcripts in PBMCs, synovial fibroblasts, synovial tissues and HeLa cells. None of the tested synovial fibroblasts expressed any transcripts for CXCR4. In PBMCs all four transcripts were amplified, whereas HeLa cells expressed transcript 1, 2 and 4. In RA synovial tissues transcripts 1 and 2 were detectable, probably due to the infiltration of lymphocytes, since in OA synovial tissues none of the transcripts was found (Fig.4C). In contrast to CXCR4, CXCR7 protein was constitutively expressed in synovial fibroblasts (Fig.4D).

We then silenced the expression of CXCR7 with siRNA (Fig.5A) and examined whether this would influence the induction of MMPs in RASF after stimulation with CXCL12 . After transfection of siRNA targeting CXCR7, no increase in the mRNA expression of MMP-1, MMP-3 and MMP-13 was seen anymore after stimulation with CXCL12 (Fig.5B). From these experiments we conclude that CXCR7 is functionally important in synovial fibroblasts and its activation by CXCL12 leads to expression of MMP-1, MMP-3 and MMP-13.

4. DISCUSSION

In the current paper we show that the high expression of CXCL12 in RASF is due to an epigenetic alteration and that high amount of CXCL12, as found in the joints of RA patients lead to increased expression of MMPs and to joint destruction.

In cancer, both DNA hypomethylation and hypermethylation have been shown to occur [30, 31]. The promoters of p16 or the DNA repair genes MLH1 and BRCA1 for instance have been shown to be silenced by increased DNA methylation in cancer cells [32]. Global hypomethylation was correlated with demethylation of repetitive sequences e.g. Sata, D4Z4, NBL2 and transposable elements e.g. LINE-1 [33, 34]. We and others have reported global hypomethylation of synovial fibroblasts in RA and CD4 T cells in SLE [2, 35]. We also showed that this global hypomethylation affects the promoter of LINE-1 transposable element in RASF and that continuous treatment of NSF with 5-azaC induced changes in gene expression that resemble the activated status of RASF such as increased production of MMP-14, CD29 and cathepsins [2]. Also in SLE, hypomethylation of specific gene promoters have been shown to contribute to the pathogenesis of the disease [35]. Similar to the approach of our current study it was shown that normal T cells treated with 5-azaC upregulated LFA1, CD70 and perforin and that the CpG islands in the promoters of these genes were hypomethylated in T cells of SLE patients [36-38]. DNA methylation of promoter sequences can change the binding of transcription factors. Transcriptional regulation studies of the CXCL12 promoter revealed the presence of E-box consensus sequences which are binding sites for multiple transcription factors such as bHLH, NFAT, SP1 and HIF1α. [39-41]. The low methylation levels observed in the CXCL12 promoter in RASF could change the binding of these transcription factors and thereby increase gene expression. In healthy fibroblasts, the CXCL12 promoter was strongly methylated in our study. In cancers, it has been shown that DNA hypermethylation of the CXCL12 promoter in colon carcinomas, mammary carcinomas and the MCF7 breast cancer cell line inhibits tumor metastasis.

In the current study basal levels of CXCL12 were higher in RASF compared to OASF. Even though these changes were not dramatic, we believe that they are of clinical significance since CXCL12 expression positively correlated with levels of CRP. CXCL12 is expressed at high levels in RA, and it has been implicated in a number of pathogenic events such as recruitment and persistence of inflammatory cells in the synovium, as well as production of cytokines and matrix degrading enzymes [16, 17, 20, 28, 42]. The role of CXCL12 in cartilage destruction was further supported by in vivo experiments which showed that CXCL12 antagonists inhibit fibroblast-induced cell infiltration in immunodeficient mice [43]. CXCL12 can signal via CXCR4 and CXCR7. Expression of CXCR4 has been detected in synovium of healthy individuals as well as in OA and RA patients [20, 44]. Controversial data is published regarding the expression of CXCR4 in synovial fibroblasts. Whereas Garcia-Vicuna et al detected CXCR4 in synovial fibroblasts, Kanbe et al did not [16, 28]. We also could not detect mRNA for CXCR4 in synovial fibroblasts of RA or OA patients. Since, pancreatic cancer cells do not express CXCR4 due to promoter hypermethylation, CXCR4 silencing could also occur by DNA methylation in synovial fibroblasts. CXCR7, previously named RDC1 was first classified as an orphan receptor since no ligand could be found [45]. Recent publications have then related this receptor to cell recruitment, migration and proliferation after binding of CXCL12 in various cell types [9, 46, 47]. In the present study, we show that functional CXCR7 is also expressed in synovial fibroblasts and that it plays a role in the induction of MMP production via CXCL12. Based on our data we hypothesize that while CXCR4 might be responsible for CXCL12 mediated cell trafficking into RA joints, CXCR7 mediates CXCL12 signaling in resident synovial fibroblasts. The exact mechanism of signaling after binding of CXCL12 to CXCR7 is not clear yet [9, 46]. Recent studies found an association between CXCR7 and the adaptor protein β-arrestin2 [48-50]. Interactions of arrestins with different receptors have been shown to facilitate activation of the MAPK cascade, a signaling pathway which is known to regulate also MMPs [51].

High production of MMPs by RASFs has been identified as major mechanism of joint destruction in RA [52]. In particular, MMP-1 and MMP-13 were reported to play a crucial role in the invasive properties of RASF as shown in the SCID mouse co-implantation model [53, 54]. Expression of MMPs is regulated by multiple pathways. DNA hypomethylation modulates expression of different MMPs in cancer cells. In OA chondrocytes the adipocytokine leptin has not only been found to be up-regulated by promoter DNA hypomethylation but also to stimulate expression of MMP-13 [55]. Also, a variety of cytokines and chemokines stimulate the expression of MMPs [56, 57]. The chemokine CXCL12 was reported to induce the expression of MMP-3 in chondrocytes and of MMP-9 in osteoclasts [16, 27]. Furthermore, increased gelatinase and collagenase activity was described after CXCL12 stimulation in synovial fibroblasts [28]. Our present data suggest that the increased collagenase activity after CXCL12 stimulation stems from increased transcription of MMP-1 and MMP-13. On the other hand, CXCL12 stimulation neither changed the quantity of MMP-2 nor of MMP-9 transcripts, pointing to indirect regulation of gelatinase activity by CXCL12. Since MMP-3 was described to contribute to the activation of pro-MMP-9 and we found that MMP-3 is more abundant in CXCL12 stimulated cells, it is probable that CXCL12 increases gelatinase activity via upregulation of MMP-3 [58, 59]. Based on the differential induction of specific MMPs after stimulation with CXCL12 it can be concluded that CXCL12 does not induce MMPs via the Activator Protein-1 (AP-1), since all of the measured MMPs contain an AP-1 site in their promoter regions. Differences among the MMP promoters have been described and include not only binding sites for different transcription factors but also variability in the number and arrangement of binding sites, which strongly increases the complexity of MMP regulation [60].

In summary, our data show that basal expression of the chemokine CXCL12 is increased in RASF due to a defect in gene regulation by DNA methylation. In RA joints, accumulated CXCL12 produced by RASF might lead to increased expression of MMPs which mediate joint destruction. In addition to the previously reported global hypomethylation, we identified

CpG island specific hypomethylation of CXCL12 which is involved in the intrinsic activation of RASF and thereby in the perpetuation of RA.

Acknowledgements: We thank Maria Comazzi and Ferenc Pataky for their excellent technical work. We also thank our collaborators Dr. C. Buckley and Dr. A. Filer for providing us with the human lung fibroblast cultures.

Financial support: This work was supported by the Articulum Fellowship Program (YR), the Institute of Arthritis Research, Epilanges, Switzerland, and by the European Community's FP6 (Autocure) and FP7 funding (Masterswitch). This publication reflects only the author's views. The European Community is not liable for any use that may be made of the information herein.

REFERENCES

1. Ospelt C, Gay S. The role of resident synovial cells in destructive arthritis. *Best practice & research* 2008; **22**(2)**:** 239-52.

2. Karouzakis E, Gay RE, Michel BA, Gay S, Neidhart M. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. *Arthritis and rheumatism* 2009; **60**(12)**:** 3613-22.

3. Wade PA. Methyl CpG-binding proteins and transcriptional repression. *Bioessays* 2001; **23**(12)**:** 1131-7.

4. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science (New York, N.Y* 2001; **293**(5532)**:** 1089-93.

5. Hewagama A, Richardson B. The genetics and epigenetics of autoimmune diseases. *J Autoimmun* 2009; **33**(1)**:** 3-11.

6. Esteller M. Epigenetics in cancer. *The New England journal of medicine* 2008; **358**(11)**:** 1148-59.

7. Petit I, Jin D, Rafii S. The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. *Trends in immunology* 2007; **28**(7)**:** 299-307.

8. Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, Allendorf DJ *et al.* CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *Journal of molecular histology* 2004; **35**(3)**:** 233-45.

9. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z *et al.* A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *The Journal of experimental medicine* 2006; **203**(9)**:** 2201-13.

10. Raggo C, Ruhl R, McAllister S, Koon H, Dezube BJ, Fruh K *et al.* Novel cellular genes essential for transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus. *Cancer Res* 2005; **65**(12)**:** 5084-95.

11. Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A *et al.* CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**(40)**:** 15735-40.

12. De Paepe B, Schroder JM, Martin JJ, Racz GZ, De Bleecker JL. Localization of the alpha-chemokine SDF-1 and its receptor CXCR4 in idiopathic inflammatory myopathies. *Neuromuscul Disord* 2004; **14**(4)**:** 265-73.

13. Gu J, Marker-Hermann E, Baeten D, Tsai WC, Gladman D, Xiong M *et al.* A 588-gene microarray analysis of the peripheral blood mononuclear cells of spondyloarthropathy patients. *Rheumatology (Oxford, England)* 2002; **41**(7)**:** 759-66.

14. Krumbholz M, Theil D, Cepok S, Hemmer B, Kivisakk P, Ransohoff RM *et al.* Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. *Brain* 2006; **129**(Pt 1)**:** 200-11.

15. Kryczek I, Wei S, Keller E, Liu R, Zou W. Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *American journal of physiology* 2007; **292**(3)**:** C987-95.

16. Kanbe K, Takagishi K, Chen Q. Stimulation of matrix metalloprotease 3 release from human chondrocytes by the interaction of stromal cell-derived factor 1 and CXC chemokine receptor 4. *Arthritis and rheumatism* 2002; **46**(1)**:** 130-7.

17. Nanki T, Hayashida K, El-Gabalawy HS, Suson S, Shi K, Girschick HJ *et al.* Stromal cell-derived factor-1-CXC chemokine receptor 4 interactions play a central role in CD4+ T cell accumulation in rheumatoid arthritis synovium. *J Immunol* 2000; **165**(11)**:** 6590-8.

18. Burger JA, Zvaifler NJ, Tsukada N, Firestein GS, Kipps TJ. Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism. *The Journal of clinical investigation* 2001; **107**(3)**:** 305-15.

19. Nanki T, Nagasaka K, Hayashida K, Saita Y, Miyasaka N. Chemokines regulate IL-6 and IL-8 production by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *J Immunol* 2001; **167**(9)**:** 5381-5.

20. Blades MC, Ingegnoli F, Wheller SK, Manzo A, Wahid S, Panayi GS *et al.* Stromal cell-derived factor 1 (CXCL12) induces monocyte migration into human synovium transplanted onto SCID Mice. *Arthritis and rheumatism* 2002; **46**(3)**:** 824-36.

21. Watanabe N, Ando K, Yoshida S, Inuzuka S, Kobayashi M, Matsui N *et al.* Gene expression profile analysis of rheumatoid synovial fibroblast cultures revealing the

overexpression of genes responsible for tumor-like growth of rheumatoid synovium. *Biochemical and biophysical research communications* 2002; **294**(5)**:** 1121-9.

22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis and rheumatism* 1988; **31**(3)**:** 315-24.

23. Ospelt C, Kurowska-Stolarska M, Neidhart M, Michel BA, Gay RE, Laufer S *et al.* The dual inhibitor of lipoxygenase and cyclooxygenase ML3000 decreases the expression of CXCR3 ligands. *Ann Rheum Dis* 2007.

24. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 2002; **18**(11)**:** 1427-31.

25. Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* 2005; **21**(21)**:** 4067-8.

26. Distler JH, Jungel A, Huber LC, Seemayer CA, Reich CF, 3rd, Gay RE *et al.* The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles. *Proceedings of the National Academy of Sciences of the United States of America* 2005; **102**(8)**:** 2892-7.

27. Grassi F, Cristino S, Toneguzzi S, Piacentini A, Facchini A, Lisignoli G. CXCL12 chemokine up-regulates bone resorption and MMP-9 release by human osteoclasts: CXCL12

levels are increased in synovial and bone tissue of rheumatoid arthritis patients. *Journal of cellular physiology* 2004; **199**(2)**:** 244-51.

28. Garcia-Vicuna R, Gomez-Gaviro MV, Dominguez-Luis MJ, Pec MK, Gonzalez-Alvaro I, Alvaro-Gracia JM *et al.* CC and CXC chemokine receptors mediate migration, proliferation, and matrix metalloproteinase production by fibroblast-like synoviocytes from rheumatoid arthritis patients. *Arthritis and rheumatism* 2004; **50**(12)**:** 3866-77.

29. Kanbe K, Takemura T, Takeuchi K, Chen Q, Takagishi K, Inoue K. Synovectomy reduces stromal-cell-derived factor-1 (SDF-1) which is involved in the destruction of cartilage in osteoarthritis and rheumatoid arthritis. *J Bone Joint Surg Br* 2004; **86**(2)**:** 296-300.

30. Kondo Y, Issa JP. Epigenetic changes in colorectal cancer. *Cancer Metastasis Rev* 2004; **23**(1-2)**:** 29-39.

31. Lin CH, Hsieh SY, Sheen IS, Lee WC, Chen TC, Shyu WC *et al.* Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer Res* 2001; **61**(10)**:** 4238-43.

32. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *The New England journal of medicine* 2003; **349**(21)**:** 2042-54.

33. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E *et al.* Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res* 2005; **33**(21)**:** 6823-36.

34. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L *et al.* Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene* 2005; **24**(48)**:** 7213-23.

35. Richardson B. Primer: epigenetics of autoimmunity. *Nature clinical practice* 2007; **3**(9)**:** 521-7.

36. Lu Q, Wu A, Richardson BC. Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs. *J Immunol* 2005; **174**(10)**:** 6212-9.

37. Kaplan MJ, Lu Q, Wu A, Attwood J, Richardson B. Demethylation of promoter regulatory elements contributes to perforin overexpression in CD4+ lupus T cells. *J Immunol* 2004; **172**(6)**:** 3652-61.

38. Oelke K, Lu Q, Richardson D, Wu A, Deng C, Hanash S *et al.* Overexpression of CD70 and overstimulation of IgG synthesis by lupus T cells and T cells treated with DNA methylation inhibitors. *Arthritis and rheumatism* 2004; **50**(6)**:** 1850-60.

39. Calonge E, Alonso-Lobo JM, Escandon C, Gonzalez N, Bermejo M, Santiago B *et al.* c/EBPbeta is a major regulatory element driving transcriptional activation of the CXCL12 promoter. *J Mol Biol* 2010; **396**(3)**:** 463-72.

40. Ceradini DJ, Gurtner GC. Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. *Trends Cardiovasc Med* 2005; **15**(2)**:** 57-63.

41. Garcia-Moruja C, Alonso-Lobo JM, Rueda P, Torres C, Gonzalez N, Bermejo M *et al.* Functional characterization of SDF-1 proximal promoter. *J Mol Biol* 2005; **348**(1)**:** 43-62.

42. Bradfield PF, Amft N, Vernon-Wilson E, Exley AE, Parsonage G, Rainger GE *et al.* Rheumatoid fibroblast-like synoviocytes overexpress the chemokine stromal cell-derived factor 1 (CXCL12), which supports distinct patterns and rates of CD4+ and CD8+ T cell migration within synovial tissue. *Arthritis and rheumatism* 2003; **48**(9)**:** 2472-82.

43. del Rey MJ, Izquierdo E, Caja S, Usategui A, Santiago B, Galindo M *et al.* Human inflammatory synovial fibroblasts induce enhanced myeloid cell recruitment and angiogenesis through a hypoxia-inducible transcription factor 1alpha/vascular endothelial growth factor-mediated pathway in immunodeficient mice. *Arthritis and rheumatism* 2009; **60**(10)**:** 2926-34.

44. Pablos JL, Santiago B, Galindo M, Torres C, Brehmer MT, Blanco FJ *et al.* Synoviocyte-derived CXCL12 is displayed on endothelium and induces angiogenesis in rheumatoid arthritis. *J Immunol* 2003; **170**(4)**:** 2147-52.

45. Law NM, Rosenzweig SA. Characterization of the G-protein linked orphan receptor GPRN1/RDC1. *Biochemical and biophysical research communications* 1994; **201**(1)**:** 458-65.

46. Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B *et al.* The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *The Journal of biological chemistry* 2005; **280**(42)**:** 35760-6.

47. Infantino S, Moepps B, Thelen M. Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. *J Immunol* 2006; **176**(4)**:** 2197-207.

48. Zabel BA, Wang Y, Lewen S, Berahovich RD, Penfold ME, Zhang P *et al.* Elucidation of CXCR7-mediated signaling events and inhibition of CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. *J Immunol* 2009; **183**(5)**:** 3204-11.

49. Kalatskaya I, Berchiche YA, Gravel S, Limberg BJ, Rosenbaum JS, Heveker N. AMD3100 is a CXCR7 ligand with allosteric agonist properties. *Mol Pharmacol* 2009; **75**(5)**:** 1240-7.

50. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP *et al.* Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proceedings of the National Academy of Sciences of the United States of America*; **107**(2)**:** 628-32.

51. Gurevich EV, Gurevich VV. Arrestins: ubiquitous regulators of cellular signaling pathways. *Genome Biol* 2006; **7**(9)**:** 236.

52. Konttinen YT, Ainola M, Valleala H, Ma J, Ida H, Mandelin J *et al.* Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Ann Rheum Dis* 1999; **58**(11)**:** 691-7.

53. Jungel A, Ospelt C, Lesch M, Thiel M, Sunyer T, Schorr O *et al.* Effect of the oral application of a highly selective MMP-13 inhibitor in three different animal models of rheumatoid arthritis. *Ann Rheum Dis* 2010; **69**(5)**:** 898-902.

54. Rutkauskaite E, Zacharias W, Schedel J, Muller-Ladner U, Mawrin C, Seemayer CA *et al.* Ribozymes that inhibit the production of matrix metalloproteinase 1 reduce the invasiveness of rheumatoid arthritis synovial fibroblasts. *Arthritis and rheumatism* 2004; **50**(5)**:** 1448-56.

55. Iliopoulos D, Malizos KN, Tsezou A. Epigenetic regulation of leptin affects MMP-13 expression in osteoarthritic chondrocytes: possible molecular target for osteoarthritis therapeutic intervention. *Ann Rheum Dis* 2007; **66**(12)**:** 1616-21.

56. Roach HI, Yamada N, Cheung KS, Tilley S, Clarke NM, Oreffo RO *et al.* Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. *Arthritis and rheumatism* 2005; **52**(10)**:** 3110-24.

57. Couillard J, Demers M, Lavoie G, St-Pierre Y. The role of DNA hypomethylation in the control of stromelysin gene expression. *Biochemical and biophysical research communications* 2006; **342**(4)**:** 1233-9.

58. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *The Journal of biological chemistry* 1992; **267**(6)**:** 3581-4.

59. Goldberg GI, Strongin A, Collier IE, Genrich LT, Marmer BL. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization,

complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *The Journal of biological chemistry* 1992; **267**(7)**:** 4583-91.

60. Benbow U, Brinckerhoff CE. The AP-1 site and MMP gene regulation: what is all the fuss about? *Matrix Biol* 1997; **15**(8-9)**:** 519-26.

FIGURE LEGENDS

Figure 1. RASF express more CXCL12 mRNA and secrete more protein than OASF. A) RASF (n=11, ■) expressed significantly more CXCL12 mRNA than OASF (n=8, ●) and NSF (n=1, ●). The mRNA expression of CXCL12 correlated with the patients CRP levels. B) ELISA was used to analyse the amount of CXCL12 release in RASF (n=9) and OASF (n=7) cell culture supernatants. RASF released significantly more CXCL12 than OASF. * $p<0.05$

Figure 2. Expression of CXCL12 is regulated by DNA methylation.

A) Computational analysis of the CXCL12 promoter region. (blue coloured regions: CpG islands) The percentage of C and G nucleotides (CG %) are shown 2000 basepairs upstream of the transcription initiation site (arrow) and exon 1. Bisulphite sequencing primers were designed to analyse region -741bp to -477bp and -244bp to +272bp of the CXCL12 promoter. B) Methylated CpG dinucleotides were found in the region -741bp to -477 of NSF (n=1). Each circle represents a methylated (black) or unmethylated (white) CpG dinucleotide. Every row represents a different clone. C) After treatment of OASF with 0 , 0.5 and 1 μM 5-azaC for 6 days, amounts of CXCL12 were determined by ELISA (n=4). 5-azaC treatment induced dose dependent release of CXCL12 (* $p < 0.05$). D) 5-azaC demethylates the analysed promoter region as shown by bisulphite sequencing (n=1 OASF, 6 clones were sequenced). E) McrBC digestion and CXCL12 promoter (-741bp to -477bp) quantitative PCR of RASF (n=11) genomic DNA. Negative correlation between CXCL12 mRNA expression and CXCL12 promoter DNA methylation was shown in RASF.

Figure 3. RASF have low levels of CXCL12 promoter DNA methylation.

A) Bisulphite sequencing of CXCL12 promoter region (-741 to -477) in RASF, OASF, NSF and lung fibroblasts. Nine to ten clones were sequenced from two independent RASF and OASF patients or from a pooled group of four RASF and three OASF patients. In addition,

three to six clones were sequenced from two NSF and one normal lung fibroblast culture. B) Summary of the data as percentage of CpG methylation between pooled fibroblasts cell types shows that RASF have significantly lower percentage of DNA methylation than OASF. C) Summary of the data as percentage of CpG methylation between individual fibroblasts cell types shows that RASF have significantly lower percentage of DNA methylation than OASF. The methylation percentage was calculated in B and C separately for each clone and the average of total number of clones was plotted in the histogram graph. * $p < 0.005$**.**

Figure 4. Overexpression of CXCL12 induces MMPs expression . A) Expression of MMP-1, MMP-3 and MMP-13 transcripts are significantly increased after stimulation of RASF with CXCL12 (n = 6). Data are presented as x fold change after stimulation relative to unstimulated cells. B) Time dependent release of CXCL12 protein in cell culture supernatants and inhibition of MMP1 expression using a CXCL12 blocking antibodies (n=3) C) Expression of the four known CXCR4 mRNA transcripts 1-4 by conventional RT-PCR. D) Western blot showing the expression of CXCR7 protein in HeLa cells and RASF obtained from 4 different patients; patient's samples were loaded twice on a 10% gel and the membrane was cut. The left side was incubated with anti-CXCR7 antibodies, the right side with anti-CXCR7 antibodies pre-incubated with CXCR7 synthetic peptide; α-tubulin (tub) served as a loading control.

Figure 5. CXCR7 is functional in SF. A) Expression of CXCR7 after silencing was measured on mRNA level (left panel) and on protein level (right panels). Western blot and FACS analysis confirmed silencing of CXCR7 after transfection with siRNA when compared to cells transfected with control siRNA. Grey filled line: rabbit IgG; black filled line: CXCR7 siRNA transfected RASF; black open line: scrambled control siRNA transfected RASF. B) MMP-1, MMP-3 and MMP-13 levels in control transfected RASF (sc) and CXCR7 silenced

RASF (siCXCR7) with and without CXCL12 stimulation (n = 7). All values are the mean and SEM.

Table 1. Characteristics of the study patients

Diagnosis	Patient Number	Age (years)	Gender	Disease duration (years)	NSAIDs	DMARDs	RF pos (>20 I.U.)
RA	1	55	f	18	+	steroids MTX hydroxychloroquine	+
	2	64	f	9	-	steroids	+
	3	70	m	4	+	steroids	+
	4	65	f	15	-	MTX	+
	5	52	f	19	-	Actemra,MTX	+
	6	69	f	28	-	Humira	+
	7	45	f	17	-	Salazopyrin,Arava	+
	8	65	m	15	-	MTX	+
	9	52	f	21	-	Arava	NA
	10	66	f	46	-	MTX	+
	11	67	f	37	-	Mabthera	+
	12	66	f	16	-	Arava, Prednison	+
	13	73	f	7	-	Prednison	+
	14	79	f	30	-	Prednison	NA
OA	1	95	f	NA	-	-	NA
	2	70	f	NA	+	-	NA
	3	53	f	NA	+	-	NA
	4	61	f	NA	-	-	NA
	5	71	m	NA	-	-	NA
	6	73	f	NA	-	-	NA
	7	70	m	NA	-	-	NA
	8	82	f	NA	-	-	NA
	9	62	m	NA	-	-	NA
	10	79	m	NA	-	-	NA
	11	72	f	NA	-	-	NA

RA: rheumatoid arthritis; OA: osteoarthritis; f/m: female/male; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying antirheumatic drugs; RF pos: rheumatoid factor positivity; MTX: methotrexate ; NA : not assessed.

Figure 1

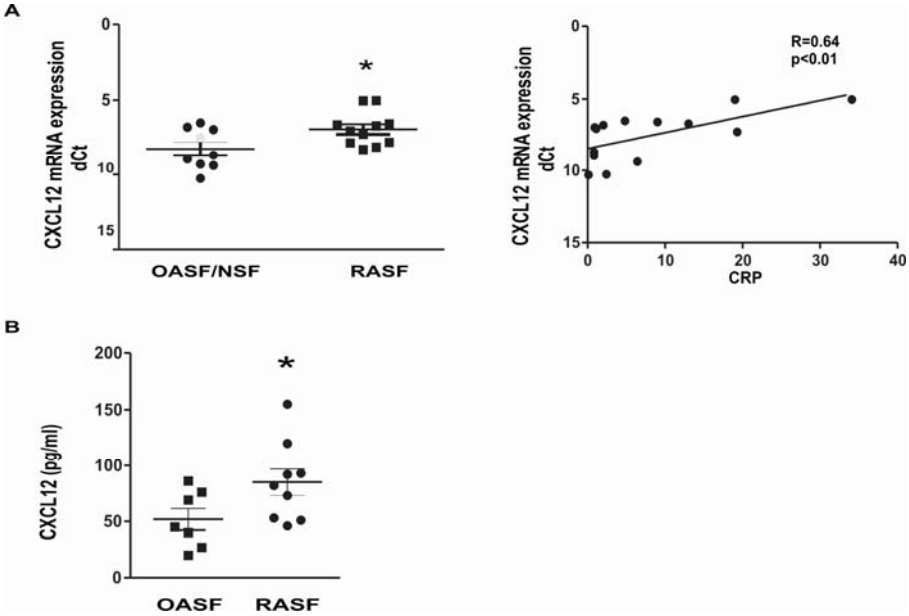


Figure 2

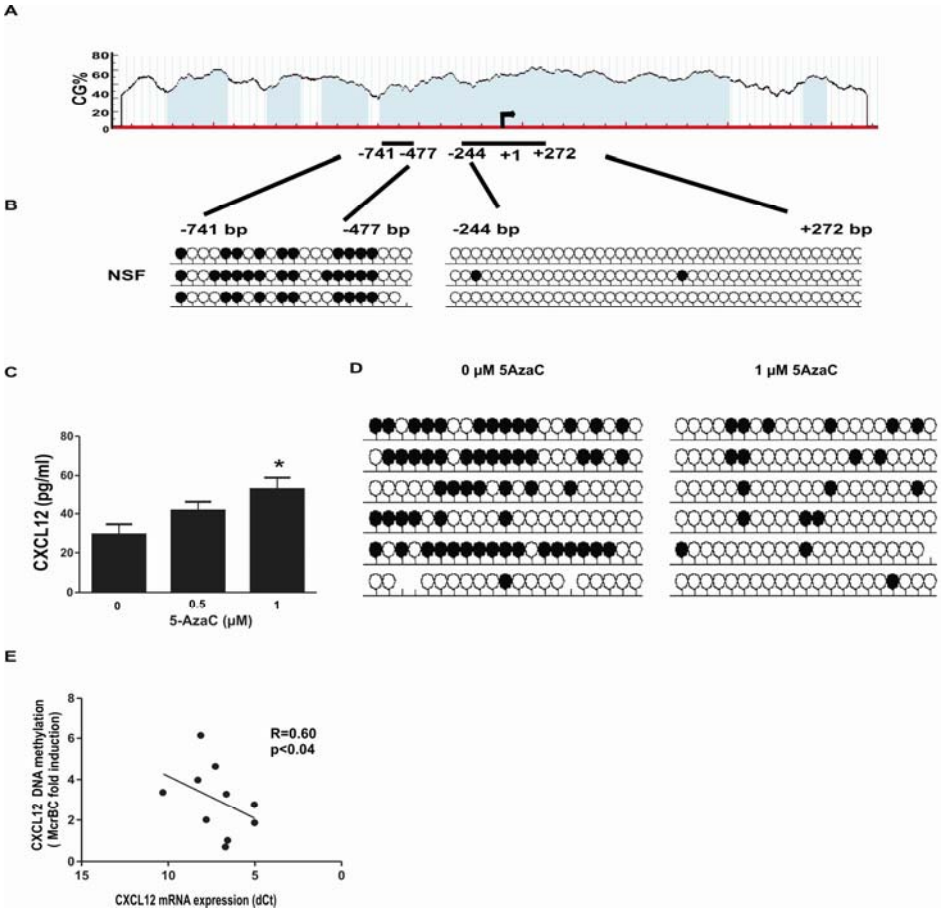


Figure 3

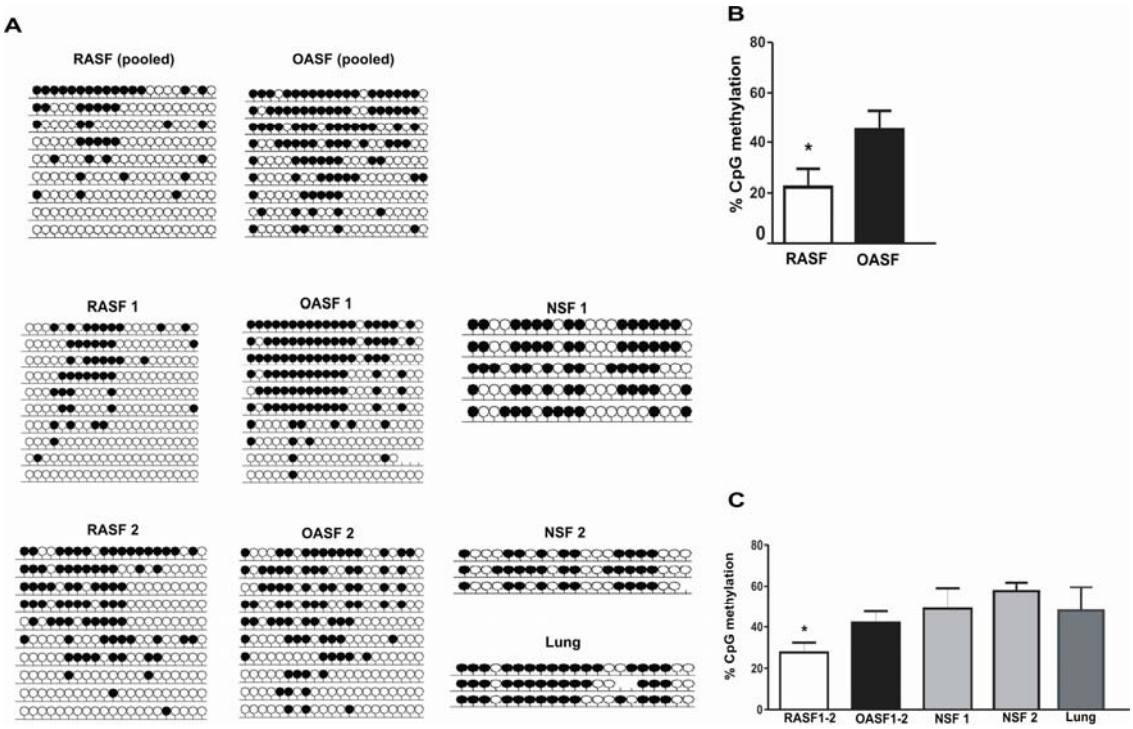


Figure 4

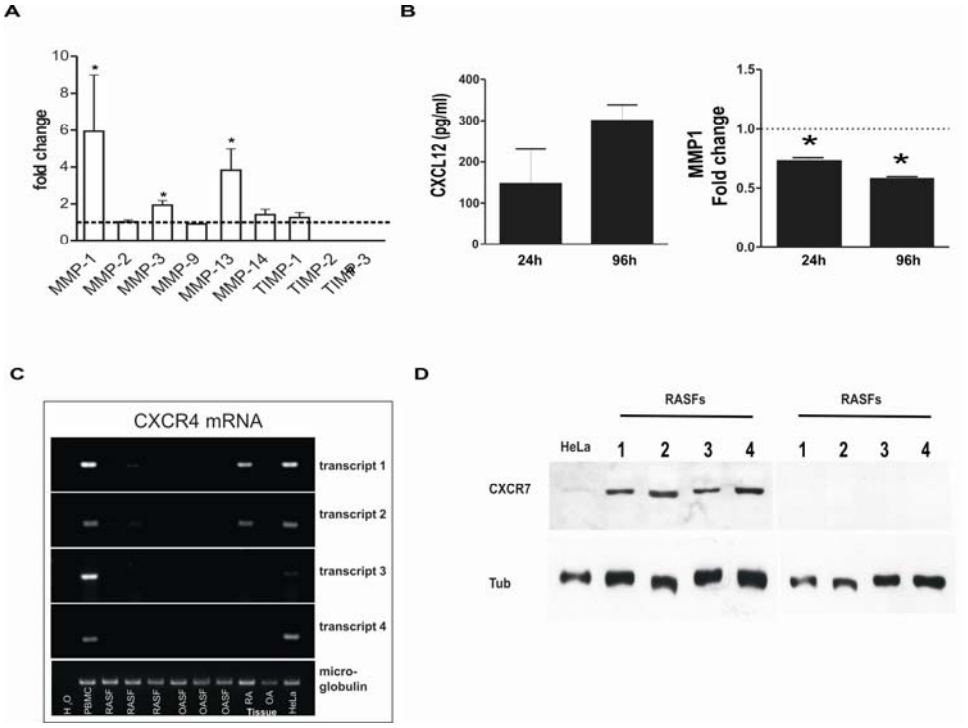
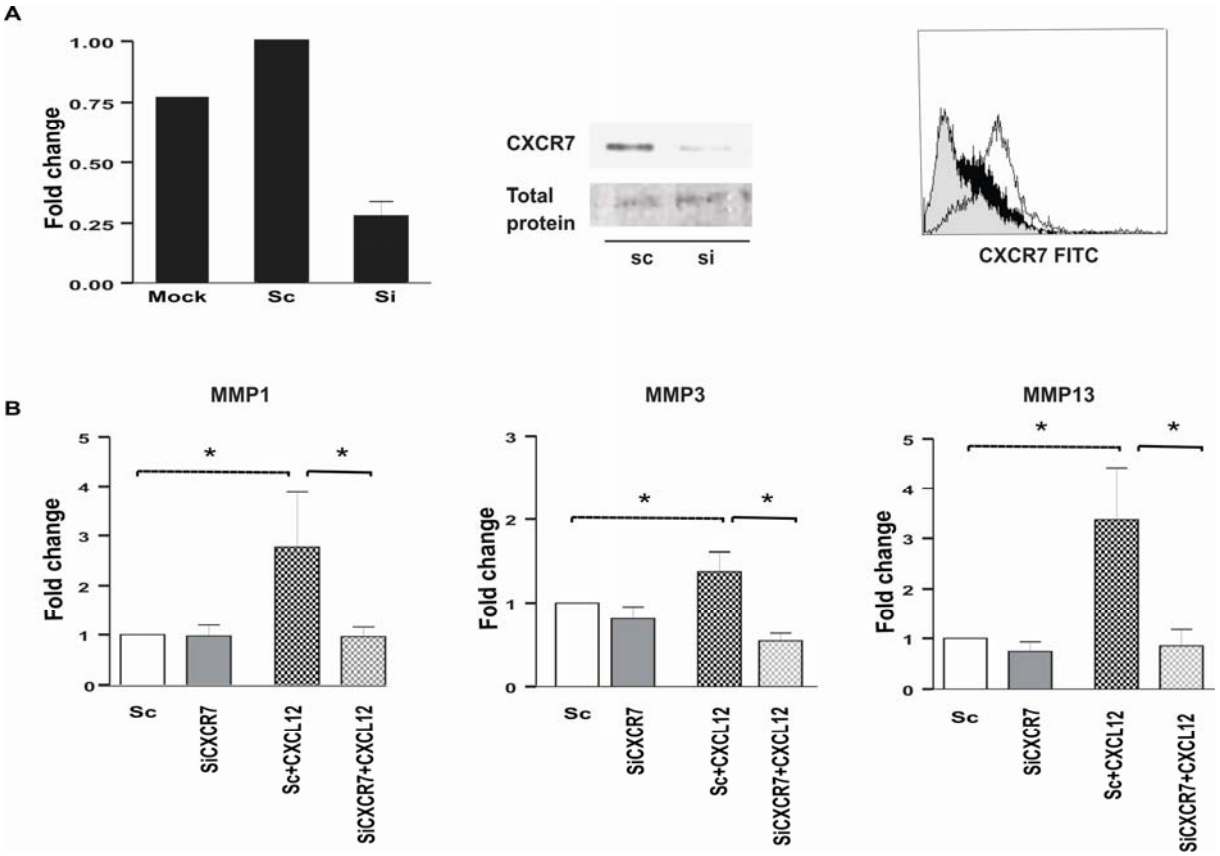


Figure 5


A
Fold change
1.00
0.75
0.50
0.25
0.00
Mock
Sc
Si
CXCR7
Total protein
sc
si
CXCR7 FITC
B
MMP1
MMP3
MMP13
Fold change
Sc
SiCXCR7
Sc+CXCL12
SiCXCR7+CXCL12
*